



Characterization and Isolation of Very Small Embryonic-like (VSEL) Stem Cells Obtained from Various Human Hematopoietic Cell Sources

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Abstract

Stem cell transplantation is one of the available treatments for leukemia, lymphoma, hereditary blood diseases and bone marrow failure. Bone marrow (BM), peripheral blood progenitor cells (PBPC), and cord blood (CB) are the predominant sources of stem cells. Recently a new type of stem cell with a pluripotent potential has been identified. These cells were named “very small embryonic like stem cells (VSELs)”. It is claimed that VSEL stem cells can be found in adult BM, peripheral blood (PB), CB and other body tissues. This study is designed to characterize and isolate VSEL stem cells from different human hematopoietic sources; CB, PB and apheresis material (PBPC). VSEL stem cells were isolated from MNC and erythrocyte layers for all materials by using centrifugation and ficoll gradient method. We determined embryonic markers by flow cytometry, immunofluorescence and western blotting methods. Results from western blotting and immunofluorescence show high level of NANOG and OCT4 protein expression in PB, apheresis material and CB. Immunofluorescence images showed cytoplasmic and nuclear presence of these proteins. Flow cytometry results exhibited a higher expression of VSELs markers on debris area than CD45-population and higher expression on CB than PB. As a result, these findings have shown that it is necessary to investigate the function of pluripotent stem cell markers in differentiated adult cells. We further conclude that erythrocyte lysis method had the highest cell recovery amount among erythrocyte lysis and ficoll gradient methods. Consequently, this study gives us new information and viewpoints about expression of pluripotent stem cell (PSC) markers in adult tissues.

Keywords Very small embryonic-like (VSEL) stem cells · Apheresis · Cord blood · Peripheral blood · Pluripotent stem cell markers

Introduction

Nowadays, stem cell transplantation (SCT) is an important treatment modality in regenerative medicine [1–3]. SCT is one of the life-saving methods frequently used in the treatment of malignant blood diseases such as lymphoma and leukemia, bone marrow deficiency and congenital blood diseases [4]. Stem cells are derived from various sources. Embryonic stem cells have a wide range of use due to their capacity to form all cells and tissues of

an adult. The use of human embryonic stem cells has been restricted in many countries around the world due to various ethical debates, problems with allogenic use and the risk of teratoma. Therefore, hematopoietic stem cell (HSC) sources are used in stem cell transplantation. For this purpose, mainly bone marrow (BM), peripheral blood progenitor cells (PBPCs) and cord blood (CB) are used [5]. Stem cells derived from these sources need to be mostly stem cells with pluripotent potential in order to provide hematopoietic regeneration [6]. After BM is collected from the donor, it is given directly (without fractionation) to the recipient. However, PBPCs and CB are passed through closed centrifugal systems where further mononuclear cell layers are isolated, stored and given to the patient when necessary.

Transplantation fails when the total amounts of CB and PBPCs are not sufficient (at least $3.5 \times 10^8 / \text{kg}$ mononuclear cells (MNC) or $2-6 \times 10^6 / \text{kg}$ CD34+) to induce hematopoietic regeneration in an adult person [7]. CB can therefore be used only in children. At the same time, the risk of Graft Versus Host Disease (GVHD) is very high at allogeneic and autologous HSC transplantation [8].

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In recent years, a new stem cell type has been identified that has the pluripotent character of human embryonic stem cells and exists throughout the entire organism [9]. The study of [10] is a pioneer for VSELs concept. These cells, which are called very small embryonic-like stem cells (VSELs), have been shown to be found in bone marrow, cord blood and peripheral blood in adults [11]. They exhibit features like high nucleus/cytoplasm ratio of 4–6 μm , expressing embryonic stem cell markers in dormancy. Kucia et al. separated the Sca-1 + / Lin- / CD45- cell group from the mouse bone marrow by FACS method. They demonstrated that this cell population synthesizes SSEA-1 and transcription factors Oct4, NANOG, Rex-1, which are not HSC markers, and which also have the quality of pluripotent stem cell marker. They reported that these cells accounted for approximately 0.02% of the bone marrow mononuclear cell population [9]. Presence of these cells, their pluripotent capacity, their ability to regenerate themselves and their in vitro/in vivo proliferation are still a matter of debate [12–14].

The amount of VSEL stem cells in peripheral blood is known to be extremely small [15]. It is thought that the amount of VSEL stem cells should be higher in aphaeresis than normal peripheral blood because of the increase in the amount of cells by using growth factors (GCSF) [16]. There is very little literature on VSELs quantity and quality of cord blood [17]. For this reason, the purpose of this study was to identify VSEL stem cells by isolating them from various sources.

Material and Methods

The samples included in the study were used with the permission of Istanbul University Clinical Research Ethics Committee dated June 20, 2013 and numbered 724. The individuals who participated in this experiment signed voluntary consent forms. Healthy 18 years and older donors were included in the study. Donors who had a chronic disease or who had been infected within the last month have not been included in the study. Since the isolation and characterization methods required working with living cells, collected blood was studied on the same day without waiting.

Cell Isolations

Cell Isolation with Ficoll

Ficoll-gradient centrifugation method is frequently used and is a method for separating blood cells into various layers [18]. In this isolation method, mononuclear cells were separated by ficoll and VSELs were purified in both the MNC layer and the erythrocyte layer. The material was diluted 1:1 with PBS (phosphate-buffered saline). The solution was added 1:2 Ficoll-

Hypaque (BioWest Lymphosep 1.077 g/mL) solution and then centrifuged at 2000 rpm for 30 min. At the end of the centrifugation, 2nd (MNC) and 4th (erythrocytes) layers were transferred to different tubes. The second layer was diluted 1:3 with RPMI (Roswell Park Memorial Institute medium) and centrifuged at 2000 rpm for 10 min. The supernatant was poured, and the remaining pellet resuspended with RPMI. Then the 4th layer, where abundant erythrocytes were present, was centrifuged at a higher speed (2690 rpm, 15 min) in the same manner. This is because VSEL stem cells are close to erythrocytes in size. While erythrocyte size is ~7–8 μm , the size of VSELs is about 5–6 μm . Thus, the supernatant was transferred to a new tube and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in RPMI after the supernatant was discarded. This suspension is the known layer of VSEL cells (5th layer) [19]. The slides were done from each layer.

Cell Isolation by Erythrocyte Lysis The other method used for the isolation of VSELs is erythrocyte lysis. Severe cell loss occurs by Ficoll method and cell loss is thought to be less in this method [20]. Therefore, both methods were used in this study. The whole material was mixed in 1: 5 ratio with the buffered solution that lysis the erythrocytes (ammonium chloride). It was kept at room temperature for 15 min. and the top layer was collected by centrifugation at 2000 rpm for 10 min. This process was repeated until the erythrocytes were completely removed. Then, by washing with PBS at 2000 rpm for 10 min, the supernatant was discarded, and the bottom pellet was suspended with RPMI. The slides were done with each layer from all samples.

Flow Cytometry Measurements In 4 different tubes, 4 alternate antibodies were examined in order to prevent the luminescence of the fluorescence. The antibodies used are SSEA-4 (stage-specific embryonic antigen 4, Santa Cruz, sc-21,704), PE anti-human CD45 (BioLegend, 304,008), FITC anti-human CD34 (BioLegend, 343,504), NANOG (Nanog homeobox, Santa Cruz, sc-30,332), CXCR-4 (CXC motif chemokine receptor 4, Santa Cruz, sc-6190), OCT 3/4 (major octamer-binding protein, Santa Cruz, sc-8628) and secondary antibodies are Goat anti-mouse IgG-TR (second antibody, Santa Cruz, sc-2781), Donkey anti-goat IgG-FITC (second antibody, Santa Cruz, sc-2024).

Flow cytometric measurements were applied only to the samples, of which erythrocytes were lysed. The lysed cells were diluted with PBS to be 1×10^6 / ml cells. 100 μl of cell suspension was placed in each flow tube. Primary antibodies were added as 1 μg in the tube and left in the dark for 30 min. At the end of the incubation, 500 μl of PBS was added to the tubes and centrifuged for 10 min. at 2000 rpm and 100 μl of PBS was applied after discarding the supernatant. Secondary antibodies were added to each tube between 0.5–1 μg and incubated for 30 min. in the dark. The supernatant was

discarded by washing with PBS. Approximately 500 μ l flow solution was added each tube for measurements.

According to the literature, the CD45 marker was negative in VSELs and CD34 was positive [21]. So, CD45- / CD34 + / SSEA-4 + cells in tube 1, CD45- / NANOG + in tube 2, CD45- / CXCR-4 + in tube 3, and CD45- / OCT-3/4+ in tube 4 was examined after gating. At the same time, since the VSEL cells were smaller than the mononuclear cells, they were also found in the debris section, and the same measurements were performed by gating the debris. The strategy of gating is shown in Fig. 1.

Immunofluorescence Staining The preparations were fixed in 96% ethanol and washed 3 times with PBS. For cell permeabilization, 0.1% triton X-100 was prepared in 2 ml PBS and incubated on ice for 15 min and for 1 h in blocking buffer (2% BSA (bovine serum albumin) and 0.3% triton X-100). The primary antibody was prepared by diluting 1:1000 in blocking solution. It was incubated overnight at +4 °C and was washed 5 \times 5 min with PBS the next day. 1:1000 dilution of secondary antibody was incubated for 1 h in the dark and was washed again with PBS 5 times of 5 min. Staining with 4 ', 6-diamidino-2-phenylindole (DAPI) (Santa Cruz

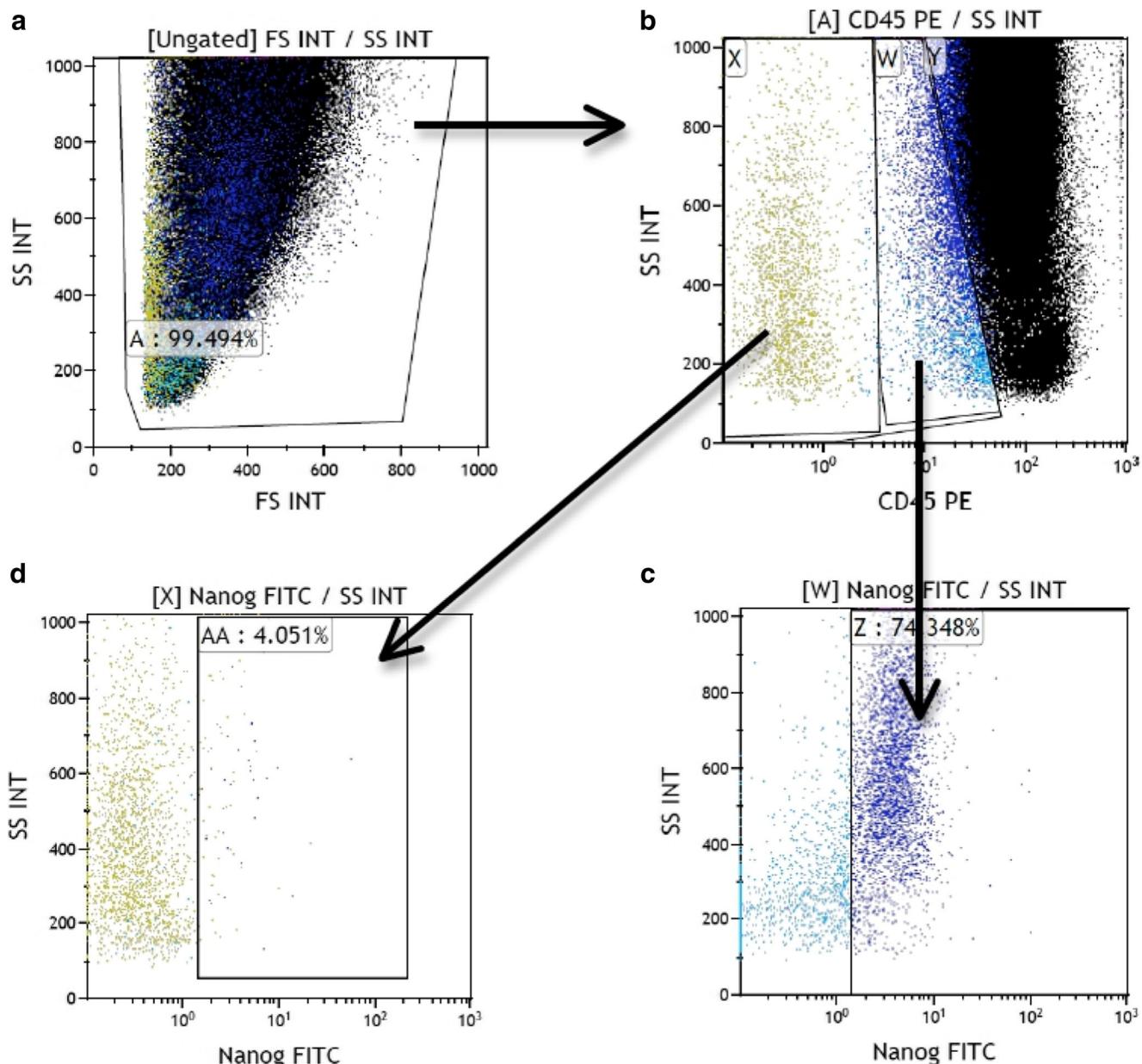


Fig. 1 Flow cytometry gating **a** shows all cell populations passing through the device. **b** The gate X shows the area of the debris and the W gate shows the area where the CD45 is negative in cells. **c** NANOG positive section is selected among the CD45 negative ones. **d** NANOG

positive section is selected among the CD45 negative ones. **d** NANOG

Table 1 Immunofluorescence preparations and antibodies

The source name	The Cell Group	Antibodies		
		1st preparation	2nd preparation	3rd preparation
Peripheral Blood Apheresis Material Cord Blood	MNC Layer Preparation	SSEA-4-TR	NANOG-FITC	OCT4-FITC
		CXCR4- FITC	CD45-PE	CD45-PE
		CD45-PE	DAPI	DAPI
	5th Layer Preparation	DAPI		
		SSEA-4-TR	NANOG-FITC	OCT4-FITC
		CXCR4- FITC	CD45-PE	CD45-PE
	Lysis Preparation	CD45-PE	DAPI	DAPI
		DAPI		
		SSEA-4-TR	NANOG-FITC	OCT4-FITC
		CXCR4- FITC	CD45-PE	CD45-PE
		CD45-PE	DAPI	DAPI

Biotechnology sc-3598) was performed and examined in fluorescence microscopy. The antibodies used for fluorescence microscopy are the same antibodies as those used in flow cytometry. The list is shown in Table 1.

Characterization of Specific Proteins by Western Blotting Method The proteins studied are NANOG and OCT4. B-actin was used as the control. Samples were centrifuged at 2000 rpm for 10 min to remove supernatants. Cell Lysis Buffer (1:10 EDTA / Tris-HCl, 0.5% Triton X-100) was prepared and 10 µl of PMSF (Phenylmethylsulfonyl fluoride protease inhibitor) stock was added to a 10 ml stock cell lysis buffer. 100 µl of lysis buffer was added to the cell pellets, incubated on ice for 30–60 min and centrifuged at 14,000 rpm for 10 min.

Protein Assay (Bradford Test) Serial dilutions were made from BSA (1.46 mg / ml) and 5 standards were obtained. Cell lysis samples were used. 10 µl of the sample and 200 µl Bradford reagent were added to the 96 wells and incubated for 5 min. Spectrophotometric measurements were utilized between 560 and 590 nm. The standard graph was done by using the measured absorbance of the standards and the amount of the proteins were determined by replacing absorbance of the samples in this graph. In this study, 12% acrylamide containing separation gel and 5% loading gel were used. The samples and 2x Laemmli Sample Buffer were mixed in a 1:1 ratio and β-mercaptoethanol was added in 5%. For protein denaturation, the samples were incubated at 80 °C for 15 min., and loaded into 20 µl gel and carried out at 130 V for 45 min. Membrane filter paper and gel system were prepared using PVDF membrane and blotted at 350 mA for 60 min.

Marking of Specific Proteins in Membrane The TBST (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) was used as a 5% milk powder blocking solution and the membrane was allowed to incubate in this solution for 1 h in the shaker. When

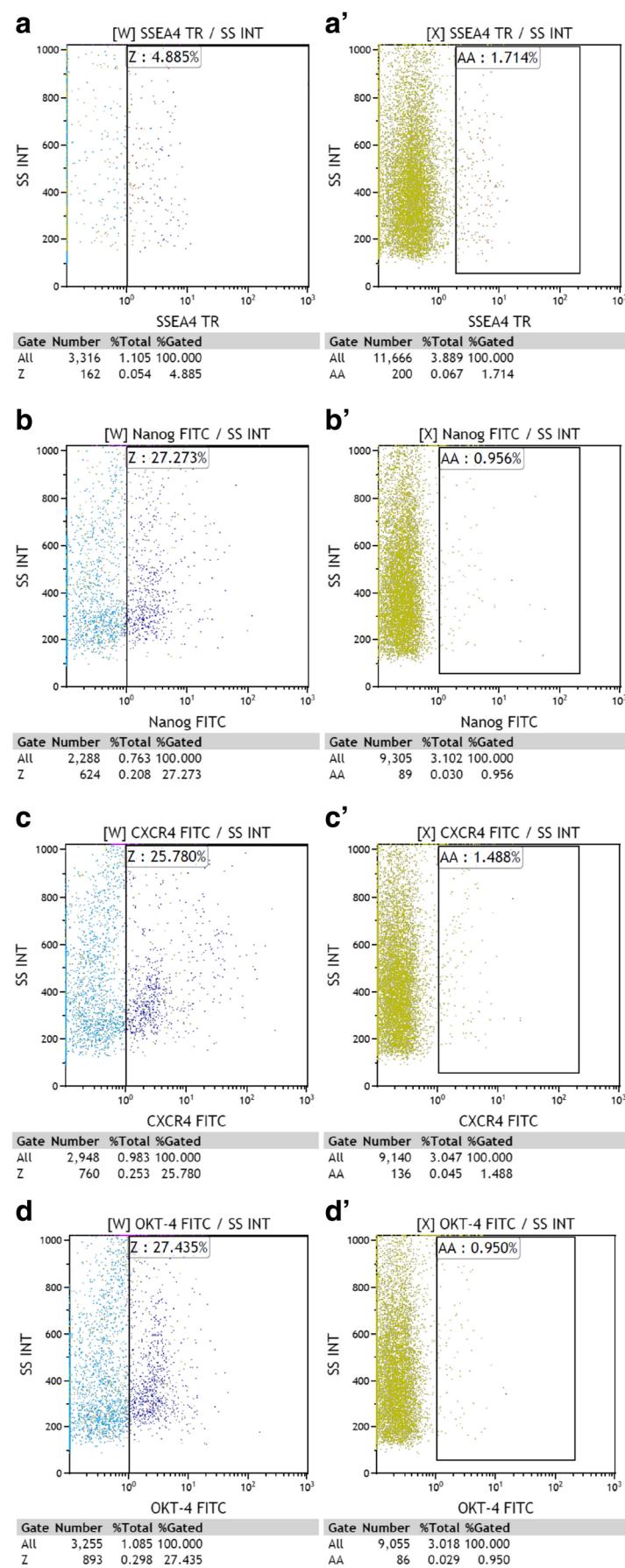
the blocking was finished, the solution was washed with TBST 3 times for 5 min. in a shaker. The primary antibody was prepared by reconstitution in the desired ratio in the blocking solution and left in the shaker at +4 °C overnight. After incubation, washing was completed with TBST 3 times for 5 min. The secondary antibody was selected as the alkaline phosphatase (AP) conjugate. Secondary antibody was incubated in 5% milk powder solution in desired ratio and incubated at room temperature for 1 h in shaker. The antibodies and dilutions used were NANOG (1:1000), OCT-4 (1:1000), β-actin (1:1000) and Donkey anti-goat IgG-AP (1:10000), respectively.

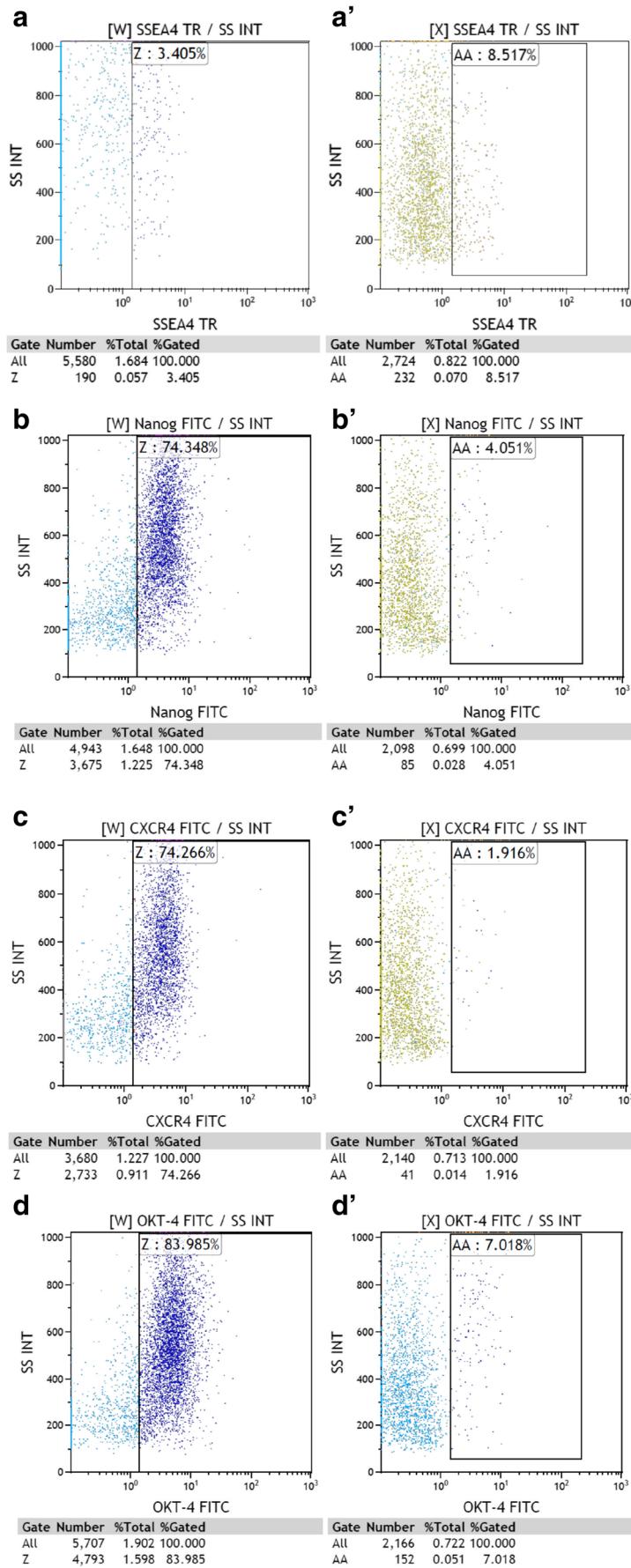
Visualization Since secondary antibody was selected as conjugated with AP, the AP substrate was screened with Novex AP Chromogenic Substrate. After the secondary antibody was washed 3 times with 5 min. TBST and rinsed with distilled water for 2 min. on the membrane shaker, Novex Substrate was placed. When the desired image was obtained, the reaction was stopped with distilled water. The membrane was dried and scanned on the computer.

β-actin was the loading control for the Western blot and used for calculating protein expression levels. Band intensities (y-axis) quantified by Image-J® software.

Statistical Analysis Graphpad Prism5 (La Jolla, America) program was used. For evaluation of flow cytometry results, “one-way ANOVA” test was performed for the antibodies in the gates and the significance of the antibodies between the

Fig. 2 VSELs markers in lysis layer obtained from peripheral blood by flow cytometry. **a** In the debris population, SSEA-4 positive cells **A'**) in the CD45- population, SSEA-4 positive cells **b** In the debris population, NANOG positive cells **B')** in the CD45- population, SSEA-4 positive cells **c** In the debris population, CXCR4 positive cells **C')** in the CD45- population, CXCR4 positive cells **d** In the debris population, OCT3 / 4 positive cells **D')** in the CD45- population, OCT3 / 4 positive cells are seen





gates was done by “two-way ANOVA” test. In Western blotting results, the values of each of the antibodies in the layers were examined by “one-way ANOVA” test. The significance of the discrepancy between the layers of two antibodies was investigated by two-way ANOVA. The results with a significance value of $p < 0.05$ were considered significant.

Results

In 8 isolated peripheral blood, 7 apheresis materials and 9 cord blood samples, VSELs markers were examined by flow cytometry, western blot and immunofluorescence staining.

In flow cytometry, two different gates were used to determine the presence and number of VSELs markers on cells. The strategy of gating were shown in Fig. 1. The flow results of a peripheral blood, an apheresis material and a cord blood sample are given in Figs. 2, 3, and 4, respectively.

In the peripheral blood samples, the correlation between the antibodies in both gates was found to be significant ($p < 0.05$). The differences in NANOG, SSEA-4 and OCT3/4 antibodies in debris and CD45- populations were found to be significant ($p < 0.05$). A comparison of the averages of the two groups is shown in Fig. 5a. In the apheresis material samples, the correlation between the antibodies in each gating was found to be insignificant ($p > 0.05$). The difference between debris and CD45- groups was shown to be insignificant ($p > 0.05$). A comparison of the averages of the two groups is given in Fig. 5b. The four antibodies between the debris and CD45- groups showed remarkably different positivity ($p < 0.05$). A comparison of the averages of the two groups is shown in Fig. 5c.

The presence of OCT3/4 and NANOG proteins was determined by western blot method in the layers of the samples (lysis, MNC and 5th layer). According to the results in the peripheral blood; OCT3/4 showed the highest expression in the lysis layer, while the least expression was shown in the 5th layer. No significant differences were observed between the layers in terms of NANOG expression. (Figure 6a). According to the results in the apheresis material; OCT 3/4 showed the highest expression in the MNC layer and the least expression in the 5th layer. NANOG expression was parallel to it. In the 5th layer, there was no difference between OCT 3/4 and Nanog expressions, while the other two layers had more

OCT3/4 expression than NANOG expression (Fig. 6b). According to the results of cord blood; OCT 3/4 showed the highest expression in the lysis layer, while the least expression was shown in the 5th layer. While no significant difference was observed between OCT3/4 and NANOG expression in the 5th layer, the expression of NANOG in the other two layers was greater than in OCT3/4 expression (Fig. 6c).

Immunofluorescence staining with embryonic markers performed in all samples of three layers were shown respectively (Fig. 7). OCT3/4 staining of single cell in lysis fraction of peripheral blood appears to localize in nucleus. Extensive staining in nucleus and cytoplasm of other samples was observed. The antibody (sc-8628 Santa Cruz Biotechnology) we use in the OCT3/4 detection is OCT4A specific and provides intranuclear staining. Nanog and SSEA4/ CXCR4 staining is observed at different levels in all samples.

Discussion

The high rate of use of hematopoietic stem cells in transplantation originates from their success in regeneration [22]. It has been suggested that there may be a non-hematopoietic, primitive group of cells in the bone marrow [23, 24]. The concept of VSELs has emerged in line with this hypothesis. The study of [25] is a pioneer for VSELs concept. In this study, they separated the Sca-1 + / Lin- / CD45- cell group from the mouse bone marrow by FACS method. They demonstrated that this cell population synthesizes SSEA-1 and transcription factors Oct4, NANOG, Rex-1, which are not HSC markers, and which also have the quality of pluripotent stem cell marker. They reported that these cells accounted for approximately 0.02% of the bone marrow mononuclear cell group [9]. In 2008, they compared the difference in peripheral blood with G-CSF mobilized peripheral blood in terms of VSELs. According to the results obtained with flow cytometry; the amount of Sca-1 + / Lin- / CD45- cell in peripheral blood was 160 cells / ml (0.0016% of total leukocyte cells), while in peripheral blood mobilized, 800 cells / ml (0.0035% of total leukocyte cells) were present. They also calculated that the expression of transcription factors such as OCT4, NANOG in mobilized blood increased according to mRNA levels. They observed OCT4 and NANOG expression in the nucleus and found that these cells do not only express pluripotent stem cell markers but also differentiate into 3 germ layers. Isolated VSEL cells formed clusters similar to cardiomyocytes, neural cells, and pancreatic cells [15]. In another study, 3 groups of lin- / CD45- / CXCR4+, lin- / CD45- / CD34+, lin- / CD45- / CD133+ cells from human cord blood were isolated. They showed that these cell groups amounted to 0.037%, 0.121% and 0.018% in total cord blood cells, respectively. Isolation of these groups via FACS lead to the observation of high levels of OCT4 and NANOG mRNA, whereas OCT4 and NANOG

Fig. 3 VSELs markers in lysis layer obtained from apheresis material by flow cytometry. **a** In the debris population, SSEA-4 positive cells **A'**) In the CD45- population, SSEA-4 positive cells **b**) In the debris population, NANOG positive cells **B')** In the CD45- population, SSEA-4 positive cells **c**) In the debris population, CXCR4 positive cells **C')** In the CD45- population, CXCR4 positive cells **d**) In the debris population, OCT3 / 4 positive cells **D')** In the CD45- population, OCT3 / 4 positive cells are seen

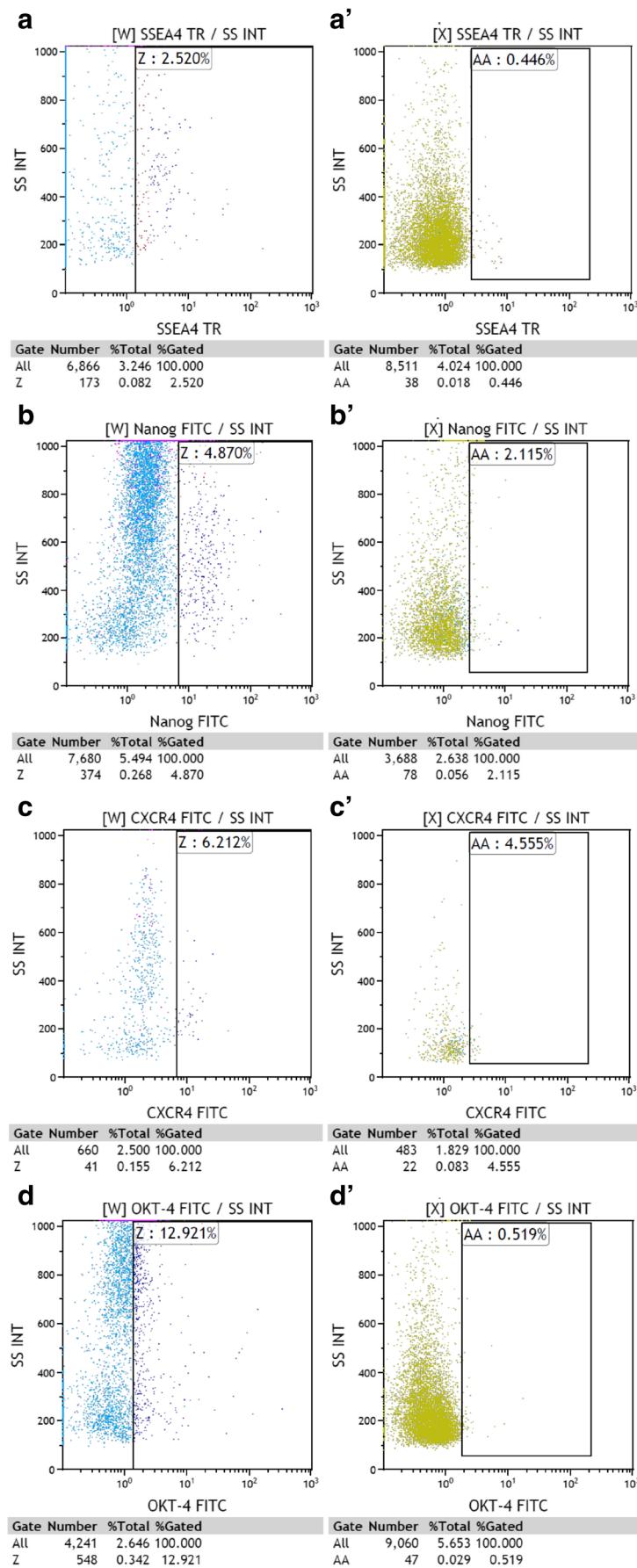


Fig. 4 VSELs markers in lysis layer obtained from cord blood by flow cytometry. **a** In the debris population, SSEA-4 positive cells **A'**) In the CD45- population, SSEA-4 positive cells **b**) In the debris population, NANOG positive cells **B')** In the CD45- population, SSEA-4 positive cells **c**) In the debris population, CXCR4 positive cells **C')** In the CD45- population, CXCR4 positive cells **d**) In the debris population, OCT3 / 4 positive cells **D')** In the CD45- population, OCT3 / 4 positive cells are seen

were observed to be localized in the nucleus and SSEA-4 on the cell surface.

Another group investigated VSEL cells differences between bone marrow and G-CSF mobilized peripheral blood (leukopheresis). In the bone marrow, 0.125% of the MNCs with Lin- / CD45- were CD34, 0.1105% were CD33 and 0.068% were CXCR4 positive. In leukopheresis, 0.034% of MNCs with Lin- / CD45- were CD34, 0.05% of CD133 and 0.042% of them were CXCR4 positive. OCT4, NANOG and SSEA-4 expressions in these cell groups were demonstrated by immunofluorescence and reverse transcriptase PCR. However, when they found that OCT4 had both nucleus and cytoplasm in immunofluorescence images, they thought that

OCT4 could have different forms [21]. In this study, the presence of VSELs in human peripheral blood, apheresis material (peripheral blood mobilized with G-CSF) and cord blood obtained by erythrocyte lysis was examined by flow cytometry. In the PB cells, 0.18% NANOG, 0.215% CXCR4, 0.235% OCT4 and 0.05% SSEA-4 were positive from CD45-group. The CD45- / Debris group showed 0.044% NANOG, 0.049%, 0.038% OCT4 and 0.101% SSEA-4 positivity ($n = 8$). In the apheresis material, it was found 0.412% NANOG, 0.304% CXCR4, 0.536% OCT4 and 0.023% SSEA-4 positive from CD45- group. 1.046% NANOG, 0.588% CXCR4, 1.253% OCT4 and 2.476% SSEA-4 were shown to be positive ($n = 3$). In cord blood, it was found to be 0.117% NANOG, 0.07% CXCR4, 0.098% OCT4 and 0.037% SSEA-4 positive. Among the CD45- / Debris group, 3993% NANOG, 3588% CXCR4, 2543% OCT4 and 0.757% SSEA-4 were shown to be positive ($n = 9$).

Bhartiya et al. (2012) determined that the cells in the 5th layer expressed OCT 4 and NANOG, and that these transcription factors were higher than those of the cells in the MNC layer. OCT4 was found to be in the cytoplasm in HSCs and in

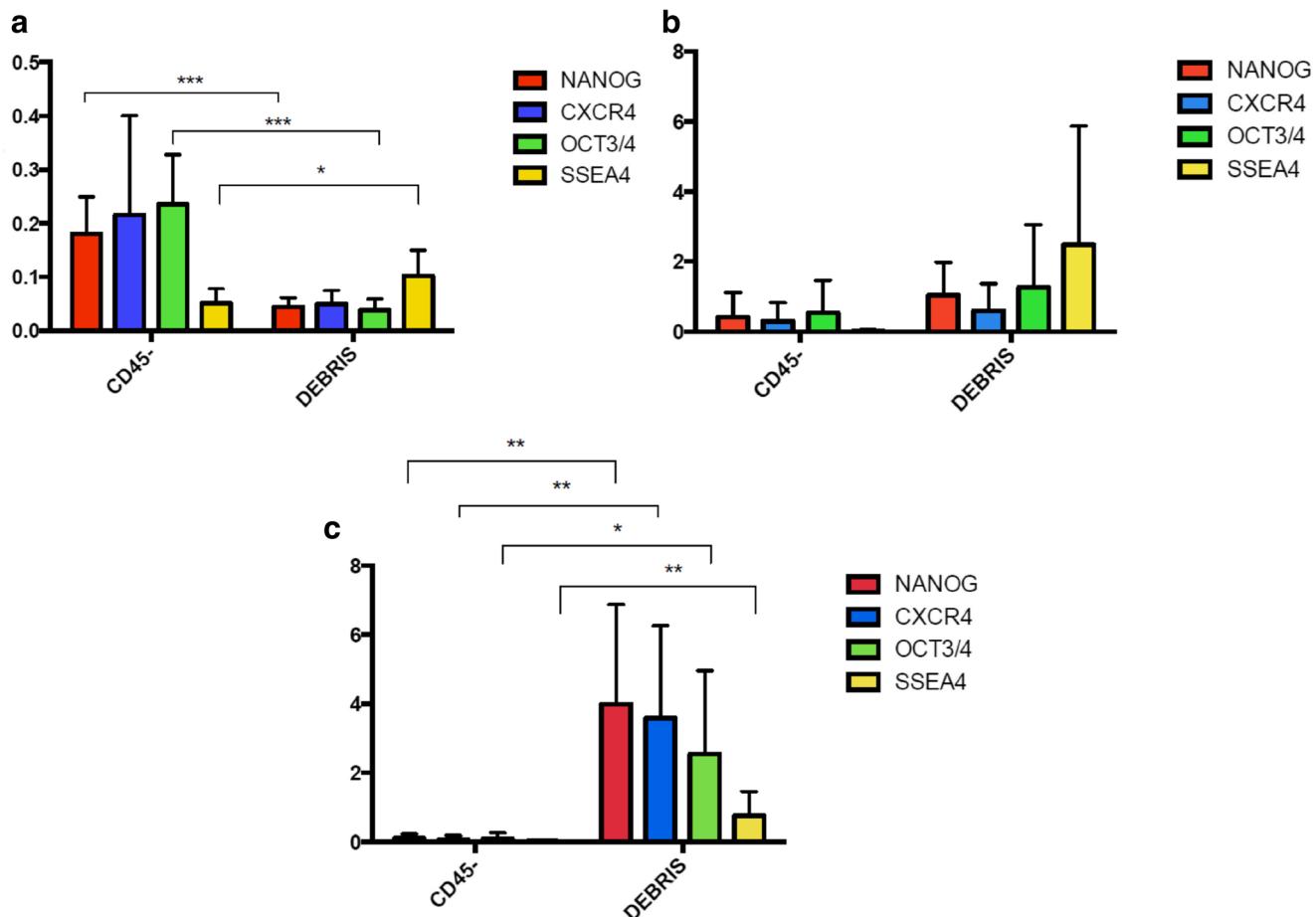
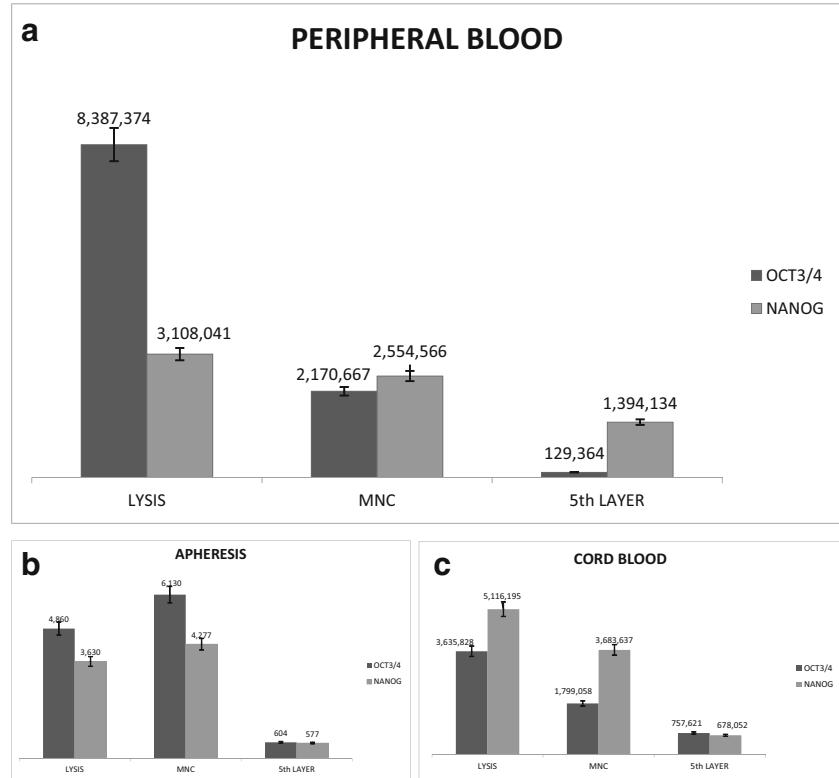


Fig. 5 Ratio of VSELs markers (NANOG, CXCR4, OCT3 / 4, SSEA-4) in CD45- populations and debris. The cells obtained from peripheral blood, apheresis material and cord blood were isolated with erythrocyte

lysing. (*) $p < 0.05$ (**) $p < 0.01$ (significance between antibodies examined by parametric t-test). **a** Peripheral blood. **b** Apheresis material. **c** Cord blood

Fig. 6 Demonstration of protein levels of VSELs markers in all cell layers by western blot (NANOG, OCT3 / 4). **a** Apheresis material **b** Peripheral blood **c** Cord blood



the nucleus in VSELs and SSEA-4 was observed to be positive in the cell surface of VSELs. They suggested that there were more VSEL cells in the erythrocyte layer than the MNC layer, and since only the MNC cells were frozen while the cord blood was stored, VSEL cells were not included in this substrate and discarded [19]. We have worked with whole and nonfrozen cord blood samples. We've found in the lysis

fractions of peripheral blood, apheresis and cord blood, similar and higher rates of embryonal markers. Therefore, these markers may be obtained in different ratios. The results were quite interesting and lead to further questions. In particular, the demonstration of the presence of a transcription factor with a pluripotent stem cell marker in peripheral blood has led us to do a more comprehensive investigation of OCT4. The results

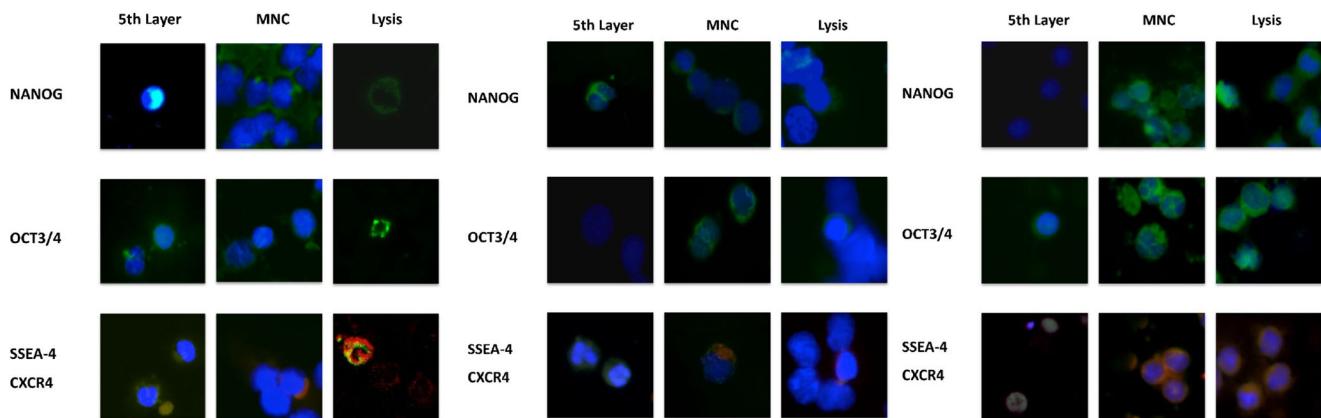


Fig. 7 Immunofluorescence of VSELs markers in cell layers (NANOG, OCT3 / 4, SSEA-4, CXCR4). Antibodies in rows and cell layers in columns are shown. It gives blue fluorescence by staining the DAPI nucleus.

The positivity of VSELs markers was indicated by green fluorescence for NANOG, OCT3 / 4 and CXCR4 and red fluorescence for SSEA-4. a) Peripheral blood b) Apheresis material c) Cord blood

in this study were similar to the findings of Zangrossi et al. (2007), who determined the presence of OCT4 in peripheral blood MNCs by PCR, Western blot and immunofluorescence methods and argued that the presence of OCT4 may be a controversial issue depending on whether it is a purely pluripotent stem cell marker or not [26]. However, another group examined the Oct4 isoforms and obtained important information about OCT 4. According to this, the OCT4 mRNA consists of 3 parts. N-terminal section, POU DNA binding site and C-terminal part. Even though the POU and C-terminal part of the OCT4A and OCT4B mRNAs are the same, the N-terminal sections are different. The OCT4B N-terminal section contains about 95 amino acids. This deficiency inhibits the ability of the OCT4B isoform to bind to DNA. In contrast to this, OCT4A can bind to the DNA and protect pluripotency. The amount of OCT4A, found in two isoforms in the embryonic cell lines, was shown to be quite high compared to the amount of OCT4B. At the same time, OCT4A is localized in the nucleus and OCT4B is seen in the cytoplasm. Furthermore, transcriptional activation by human OCT-4A was not inhibited by co-expression of OCT-4B [25]. The exact function of the OCT4B isoform, in the cells it is present, and its interactions with OCT4A are unknown. This remains a topic not yet elucidated and requires more extensive research.

In our immunofluorescence staining, OCT4A was observed only in a single cell in lysis fraction of peripheral blood and appears to be localized in nucleus, as these cells are very rare, especially in peripheral blood. It was observed in cytoplasm and nucleus of other samples. The antibody (sc-8628 Santa Cruz Biotechnology) we use in the OCT3/4 detection is OCT4A specific and provides intranuclear staining [27–29]. We think that Oct4A expression are present in the western blot results. Immunofluorescence images also support this argument. Considering the Western blot results, we think that it is not wrong to say that the OCT4A isoform is expressed in large amounts in PB, mobilized PB and CB.

NANOG is an important transcription factor in providing pluripotency. In combination with OCT4 and Sox2 genes, the cell remains pluripotent. NANOG ensures that the pluripotent property is maintained, and when the amount of NANOG decreases, the pluripotential property decreases. In this study, a high NANOG expression was observed in mobilized PB and CB, whereas a much less NANOG expression was observed in PB. However, it was also observed that NANOG antibody produced more than one band in western blot results and they have in different MW. This suggests that NANOG degrades rapidly and gives a band sequence by post translational modifications [30]. Although its presence in PB is controversial, a high rate of expression in CB and mobilized PB suggests the same question for NANOG, whether NANOG is specifically a stem cell marker or not. [31]. According to the results of Western blotting, it was claimed that multiple bands of NANOG protein could be due to post-translational

modifications [30]. It has been shown that 3 different NANOG proteins are formed in mouse. It was reported that b variants may be caused by the ladder form that appears in the western blot. [32]. It can be said that NANOG protein is found in peripheral blood in this study. Especially in mobilized PB expression is high. According to the results, NANOG protein forms a ladder. So, we thought that it is a difficult antibody to optimize. It was determined that the bands have different weights in certain layers. It was found that NANOG protein has different cellular localization in varied cell types [30]. The presence of NANOG in human cervical cancer cells has been shown to be cytoplasmic and NANOG cellular localization has been reported to vary according to cell type and stage of cancer [33]. The presence of NANOG in this study was cytoplasmic in all layers in PB and mobilized PB; but both nucleic and cytoplasmic were shown in the CB. These data suggest that the cellular localization of the NANOG protein may be changing, perhaps with the primitive primer of the cell. As cell progresses from primitivity to differentiation, it is thought that the presence of NANOG protein changes from nucleus to cytoplasm. So far, the presence of NANOG has been demonstrated in embryonic stem cells, primordial germ cells, human germ cell tumors, mesenchymal stem cells, testicular cancer, breast carcinomas, fetal testis, malignant cervical epithelial cells and adult human fibroblasts [33–37].

In general, we did not find enough cells with high regeneration capacity in the 5th layer as shown by Bhartiya and his team [19]. Although there are pluripotent cells in the 5th layer, it is thought that they will not be in sufficient quantity and that they will not increase the success rate of transplantations. Further research is required to fully understand the character of VSEL cells. At the end if the capacity of these cells to renew themselves, the tissues they are found and the functions they have are understood, this could give way to the potential use in clinic.

Conclusion

According to western results, high levels of OCT3/4 and Nanog that have embryonic markers were detected in the lysis and MNC layers of peripheral blood and apheresis material. The OCT3/4 protein is most abundant in the peripheral blood lysis layer compared with all samples, and is compatible with immunofluorescence picture, although it appears in a small number of cells. A similar but lesser ratio is found in the 5th layer obtained from apheresis and cord blood. It has been difficult to compare embryonic markers between samples and layers and to evaluate them correctly due to lack of literature. The high number of embryonic markers in the lysis layer of all samples may be due to the presence of both MNC and VSELs in this layer. Because flow cytometry analysis requires a large number of cells, all samples can be made in

lysis layers. In the analysis of debris and CD45 negative cells, we found a small number of NANOG, CXCR4, OCT3 / 4 and SSEA-4 positive cells in all samples.

As a result, VSELs isolation with erythrocyte lysis from all hemopoietic samples may provide a higher rate of cell yield expressing embryonic markers for hematopoietic regeneration.

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Compliance with Ethical Standards

Conflict of Interest All authors declare no conflicts of interest.

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